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Pathways for Fatty Acid Elongation and Desaturation in *Neurospora crassa*

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ABSTRACT: *Neurospora crassa* incorporated exogenous deuterated palmitate (16:0) and ^{14}C-labeled oleate (18:1$^{\Delta 9}$) into cell lipids. Of the exogenous 18:1$^{\Delta 9}$ incorporated, 59% was desaturated to 18:2$^{\Delta 9,12}$ and 18:3$^{\Delta 9,12,15}$. Of the exogenous 16:0 incorporated, 20% was elongated to 18:0, while 37% was elongated and desaturated into 18:1$^{\Delta 9}$, 18:2$^{\Delta 9,12}$, and 18:3$^{\Delta 9,12,15}$. The mass of unsaturated fatty acids in phospholipid and triacylglycerol is 12 times greater than the mass of 18:0. Deuterium label incorporation in unsaturated fatty acids is only twofold greater than in 18:0, indicating a sixfold preferential use of 16:0 for saturated fatty acid synthesis. These results indicate that the release of 16:0 from fatty acid synthase is a key control point that influences fatty acid composition in *Neurospora*.
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The fatty acid composition of lipids determines their structural and functional properties. However, the biochemical control of fatty acid composition is not well understood. The use of model systems has provided considerable insight in elucidating fatty acid and glycerolipid metabolism.

The filamentous fungus *Neurospora crassa* has advantages as a model system for comparison to plants: it is one of few well-studied microbes able to produce the polyunsaturated fatty acids *de novo* that plants also make (1). It is also capable of incorporating exogenous fatty acid into its lipids (2–4). In addition, several mutants with defects in the synthesis of fatty acids and glycerolipids (3–7) have been described, and the lipid composition of wild type and mutants has been examined under varying conditions of temperature, medium, and fatty acid supplementation (2–4,8–10).

In *Neurospora*, as in plants, the pathway for biosynthesis of the major fatty acids begins with formation of 16:0 from acetyl-CoA and malonyl-CoA. In the major pathway of fatty acid biosynthesis, other fatty acids are derived from 16:0: 16:0 is first elongated to stearate (18:0), which undergoes consecutive desaturations to form 18:1$^{\Delta 9}$, 18:2$^{\Delta 9,12}$, and 18:3$^{\Delta 9,12,15}$ (1). We have examined the metabolism of exogenous palmitate (16:0) and oleate (18:1$^{\Delta 9}$) by *Neurospora* to better understand the regulation of fatty acid biosynthesis and control of fatty acid composition.

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EXPERIMENTAL PROCEDURES

Growth of cultures, fatty acid-supplementation conditions, and label incorporation. Neurospora crassa wild-type strain 74-OR8-1a was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center (Kansas City, KS). Conidia (1×10^5) were inoculated into culture flasks containing 50 mL of liquid Vogel's medium N (5) and incubated at 34°C with shaking for 24 h. The concentration of labeled fatty acids was 30 μM: Deuterated 16:0's [7,7,8,8-$^2H_4$]16:0; [2,2-$^2H_2$]16:0, [16,16,16-$^2H_3$]16:0, all 98% purity, Cambridge Isotope Laboratories (Andover, MA) were not diluted, and [1-^{14}C]18:1$^{\Delta 9}$—86.4 kBq [^{14}C]18:1$^{\Delta 9}$, 1.11-2.22 GBq/mmol, ICN Pharmaceuticals, Inc. (Costa Mesa, CA) was diluted with unlabeled 18:1$^{\Delta 9}$ (Sigma, St. Louis, MO). Cultures were incubated for another 24 h before lipid extraction. Under these conditions, addition of the fatty acid had no apparent effect on rate of growth or final culture weight.

Lipid analysis. Lipids were extracted from cultures, fractionated on Sep-Pak silica columns to yield neutral lipids, phospholipids, and glycolipids; the triacylglycerols were separated from other neutral lipids by thin-layer chromatography as previously described (5). Radiolabel incorporation into lipid fractions was determined by scintillation counting of aliquots.

Fatty acids were analyzed as fatty acid methyl esters by gas chromatography with flame-ionization detection as described (5), using methyl heptadecanoate as internal standard. The column was a fused silica capillary column (30 m × 0.25 mm) coated with Stabilwax (df = 0.2 μm) (Restex, Bellefonte, PA). Label incorporation into fatty acids of ^{14}C-labeled samples was monitored by high-performance liquid chromatography of fatty acid methyl esters as in Reference 11.

Gas chromatography–mass spectrometry (fused silica capillary, 20 m by 0.18 mm, coated with DB-WAX and df = 0.3 μm; J&W Scientific, Folsom, CA) was used to confirm the identity of fatty acid derivatives and to determine the metabolic fate of deuterated 16:0 (5). Identification was based on comparison of spectra from sample runs to those of standards and published spectra.

The metabolic fate of [7,7,8,8-$^2H_4$]16:0 was followed by operating the mass spectrometer in the selected ion monitoring mode to monitor the M^+ and $(M + 4)^+$ ions of fatty acid

methyl esters from C_{14} to C_{24}, as well as the $(M + 2)^+$ ions for C_{18} unsaturated fatty acids (since desaturation at the 9,10-position following elongation to 18:0 leads to loss of two of the ^2H). The fraction of each fatty acid which was deuterated was calculated from peak areas for the M^+ and $(M + 4)^+$ or $(M + 2)^+$ ions for each fatty acid methyl ester. Correction was made for the fraction of $(M + 2)^+$ ions present due to normal isotope abundance in fatty acid standards (Alltech, Deerfield, IL). A similar procedure was used for 16:0 deuterated at other carbons.

Double-bond location in the monounsaturated fatty acids was determined from mass spectrometry of pyrrolidide derivatives prepared from fatty acid methyl esters (12).

RESULTS AND DISCUSSION

Supplementation of *Neurospora* cultures with 30 μM $18:1^{\Delta 9}$ or 16:0 had only a minor effect on the relative fatty acid composition of lipids, as shown in Figure 1A, B, and C. In previous studies (2–5), *Neurospora* lipids were enriched in the supplemented fatty acid, although these studies used higher concentrations of fatty acid than our study. We observed slightly increased concentrations of unsaturated fatty acids for $18:1^{\Delta 9}$-supplemented and of saturated fatty acids for 16:0-supplemented cultures, compared to unsupplemented cultures.

Over 97% of the exogenous [1-^{14}C]$18:1^{\Delta 9}$ was taken up by the cultures, and 59% of that taken up was converted to $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$ (Fig. 2A), and to a lesser extent (1–2% of total label), to $20:1^{\Delta 11}$, a minor fatty acid in *Neurospora*. Thus, the added $18:1^{\Delta 9}$ was readily desaturated and a small amount elongated. Specific activities (cpm/mmol) of the individual ^{14}C-labeled fatty acid differed (Fig. 2A); in phospholipid, which includes the phosphatidylcholine substrate for $18:1^{\Delta 9}$ and $18:2^{\Delta 9,12}$ desaturation, the specific activity of $18:2^{\Delta 9,12}$ was 0.8×10^9 cpm/mmol while the specific activities of $18:1^{\Delta 9}$ and $18:3^{\Delta 9,12,15}$ were greater (by 73 and 112%, respectively). This difference suggests preferential use of the [^{14}C]$18:2^{\Delta 9,12}$ for desaturation. The proportion of ^{14}C-label incorporated into each lipid class was similar to the proportion of its fatty acid mass, with the exception of the lower amount of label vs. mass in the glycolipid fraction (Table 1).

Cultures supplemented with [7,7,8,8-$^2H_4$]16:0 incorporated the deuterated fatty acid, with different levels of label incorporation in individual fatty acids (Fig. 2B). Twenty percent of the [^{2}H]16:0 was elongated to [^{2}H]18:0 as an end product, and both labeled compounds were incorporated into triacylglycerol. Further elongation of 18:0 to longer-chain saturated fatty acid (20:0, 22:0, and 24:0) in triacylglycerol and phospholipid was similarly efficient, with label in 20–22% of the longer-chain fatty acid. The relative proportion of [^{2}H]-labeled fatty acid in the lipid classes differed from the fatty acid mass (Table 1); a lower amount of label was incorporated into phospholipids, and a higher amount into triacylglycerol. This observation is consistent with the role of triacylglycerol as storage lipid that can accommodate a higher relative level of saturated fatty acid than phospholipids, which are desaturation substrates and, as components of membranes, appear to regulate fatty acid composition more strictly (9).

In contrast to the efficient incorporation of label from [^{2}H]16:0 into saturated fatty acids, label was relatively poorly incorporated into the major unsaturated fatty acids (Fig. 2B). Although the unsaturated fatty acids were present at 12 times the mass of 18:0 in the phospholipid and triacylglycerol, they contained only 37% of the incorporated [^{2}H]16:0. The lowest

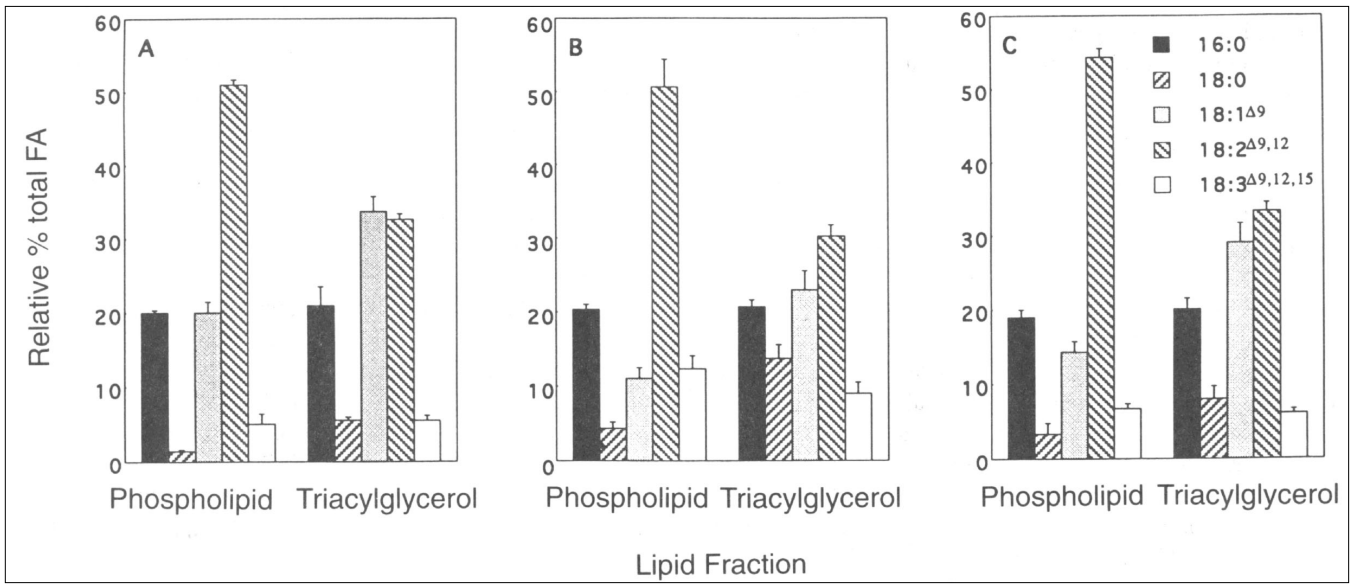

FIG. 1. Fatty acid (FA) composition of *Neurospora* lipids. A, 50 mL cultures supplemented with 30 μM [1-^{14}C]$18:1^{\Delta 9}$ (specific activity 3.3×10^9 cpm/mmol) as described in the Experimental Procedures section; B, cultures supplemented with 30 μM [7,7,8,8-^2H]$_4$16:0; C, unsupplemented cultures. Values are means ± SE of three or more experiments. (Balance in minor fatty acids contributes 2% or less of the total.)

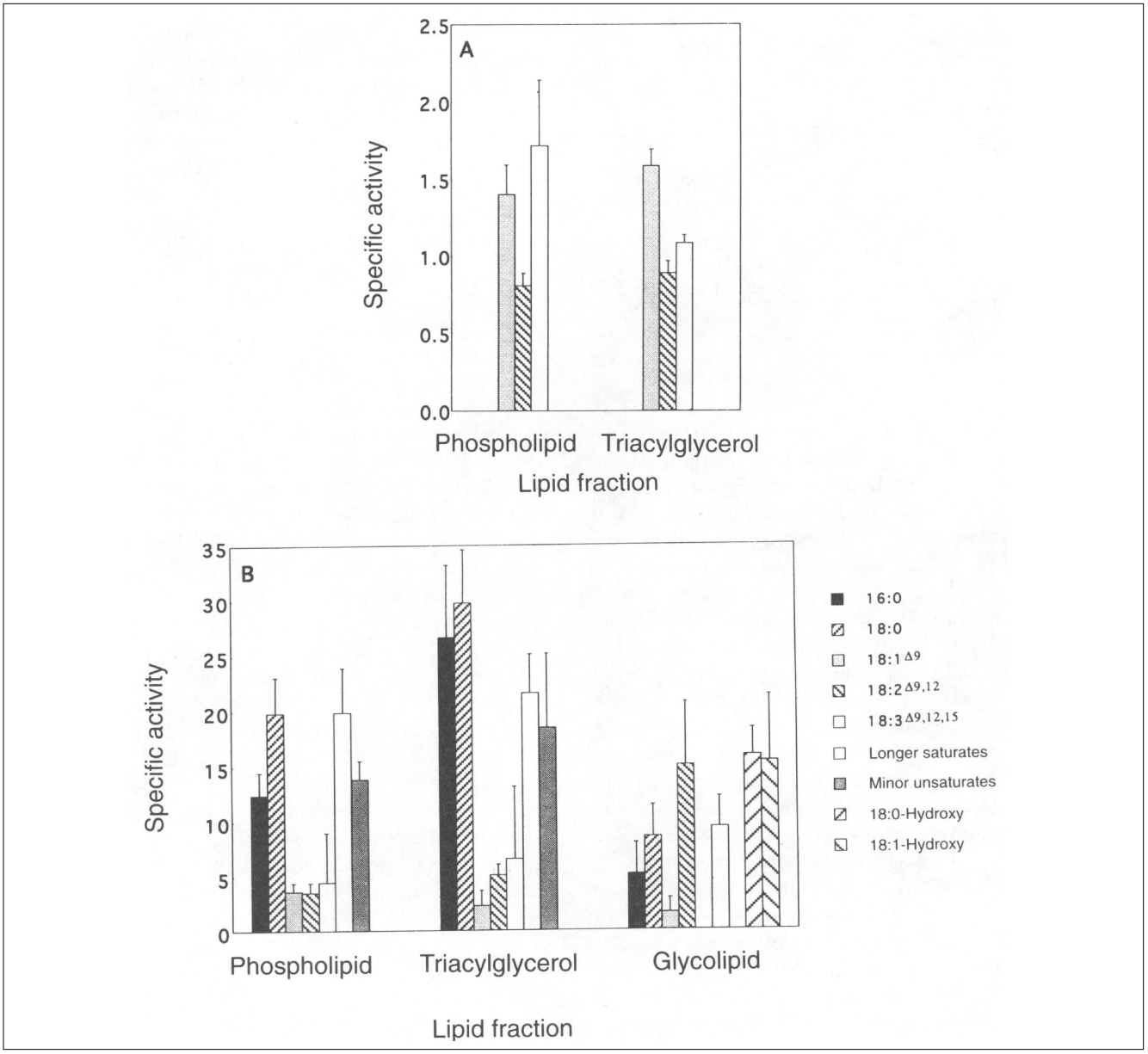

FIG. 2. Specific activity of fatty acids in *Neurospora* lipids. A, Cultures supplemented with 30 μM [1-^{14}C]18:1$^{\Delta 9}$ (label incorporation into glycolipids not detectable). Samples were analyzed for label incorporation by high-performance liquid chromatography and for fatty acid mass by gas chromatography with flame-ionization detection. Values are average specific activities (10^9 cpm/mmol) for six samples; error bars indicate SE calculated from normalized values. B, Cultures supplemented with 30 μM [7,7,8,8-$^2H_4$]16:0. Deuterium incorporation determined by gas chromatography/mass spectrometry with selective ion monitoring. Values are average specific activity (% deuterated) ± SE for three experiments. Long-chain saturated fatty acids, average for 20:0, 22:0, 24:0; minor unsaturated fatty acids, 16:1$^{\Delta 9}$ and 18:1$^{\Delta 11}$. Missing values (both panels) indicate that insufficient material was present to calculate specific activity.

level of deuteration was found in 18:1$^{\Delta 9}$. In phospholipid, the percentage deuterated 18:0 (specific activity, Fig. 2B) is five times greater than the percentage deuterated 18:1$^{\Delta 9}$. This five-fold dilution must occur as the result of endogenously produced 18:1$^{\Delta 9}$, indicating that desaturation of 18:0 to 18:1$^{\Delta 9}$ is more efficient for 18:0 synthesized *de novo* by the fatty acid synthase. The polyunsaturated fatty acids had higher levels of deuteration than 18:1$^{\Delta 9}$, indicating that once 18:1$^{\Delta 9}$ is formed, it is readily used by the desaturation pathway. The lower level of deuterated unsaturated fatty acids formed from the exogenous 16:0 is not due to inhibition of desaturation by [^{2}H]16:0, since the fatty acid composition of these cultures is similar to

TABLE 1
Relative Amounts of Total Fatty Acid in Lipid Fractions of *Neurospora* Cultures[a]

Supplement	Lipid fraction	Total fatty acid mass[b] (%)	Total label[b] (%)
[1-^{14}C]18:1$^{\Delta 9}$	Phospholipid	74.03 ± 4.20	71.97 ± 4.09
	Triacylglycerol	16.28 ± 3.67	18.98 ± 4.44
	Glycolipid	0.95 ± 0.30	0.26 ± 0.02
[7,7,8,8-$^{2}H_4$]16:0	Phospholipid	76.80 ± 5.89	58.81 ± 9.12
	Triacylglycerol	18.63 ± 1.03	35.07 ± 9.11
	Glycolipid	1.87 ± 0.34	3.15 ± 1.41

[a]Total fatty acid, [^{14}C]18:1$^{\Delta 9}$-supplemented, 911 µg/culture (average of 6); [^{2}H]16:0-supplemented, 1005 µg/culture (average of 3).
[b]Balance of material in remaining neutral lipids, including free fatty acid for 18:1$^{\Delta 9}$.

that of cultures grown without exogenous [^{2}H]16:0 (Figure 1B and 1C). A primary isotope effect that inhibits desaturation of only the deuterated 18:0 is possible, because the elongation product of [7,7,8,8-$^{2}H_4$]16:0 would be the [9,9,10,10-$^{2}H_4$]18:0. However, a similarly low specific activity of 18:1$^{\Delta 9}$ relative to its precursors and products was also observed when wild-type *Neurospora* cultures were supplemented with [2,2- or 16,16,16-^{2}H]16:0 (Table 2) or [1-^{14}C]16:0 (13). Since exogenous, deuterated 16:0 is efficiently elongated to 18:0, while the deuterated 18:0 produced is not efficiently desaturated to 18:1$^{\Delta 9}$, we conclude that elongation of the exogenous 16:0 to 18:0 occurs in a metabolic pool separate from that of *de novo* fatty acid synthesis.

[^{2}H]16:0 was also converted into palmitoleate (16:1$^{\Delta 9}$) and *cis*-vaccenate (18:1$^{\Delta 11}$) (Fig. 2B), products of a minor desaturation pathway in *Neurospora* (3). The 16:1$^{\Delta 9}$ results from formation of 16:0-CoA and desaturation, probably by the stearoyl-CoA Δ9-desaturase, which inserts a double bond in the 9,10-position of the fatty acid chain. The yeast and animal forms of this enzyme are capable of desaturating 16:0-CoA (14). Subsequent elongation of 16:1$^{\Delta 9}$ produces 18:1$^{\Delta 11}$.

Glycolipids are a relatively minor, though important, component of *Neurospora* lipids. Although 24-carbon saturated hydroxy fatty acids have been identified as the major components of *Neurospora* glycosphingolipids (15), we found that 60–70% of the total glycolipid fatty acid was C_{18} hydroxy fatty acid (2-hydroxystearate and 2-hydroxyoctadecenoate) and about 10% was C_{20}–C_{24} hydroxy fatty acid, with the remaining fatty acid not hydroxylated. Label from [^{2}H]16:0 was readily incorporated into both C_{18} hydroxy fatty acids (Fig. 2B). [^{2}H]16:0 incorporation into glycolipids was proportionally higher than [^{14}C]18:1$^{\Delta 9}$ incorporation (Table 1), presumably due to the 2-hydroxy fatty acid in this fraction being derived from elongation products of 16:0.

The percentage deuterated fatty acid (specific activity, Fig. 2B) present in saturated fatty acids ranged from 20–30%, and in minor fatty acids is 14–18%, while it ranged from 2–6% for the major unsaturated fatty acids. Thus, the exogenously added [^{2}H]16:0 was less efficiently utilized for the major desaturation pathway. We infer that the [^{2}H]18:0 produced by elongation of [^{2}H]16:0 is not as accessible to desaturation as 18:0 formed *de novo*. Therefore, 18:0 derived from exogenous [^{2}H]16:0 behaves as if in a separate metabolic pool. Our evidence is consistent with two pathways of biosynthetic reactions leading from 16:0. One is the main pathway leading to unsaturated fatty acids, originating from the fatty acid synthase reaction *via* elongation of 16:0 to 18:0, then desaturation to 18:1$^{\Delta 9}$. The other pathway leads to accumulation of saturated fatty acids. These appear to be formed primarily after thioesterase hydrolysis of the 16:0 fatty acid synthase product to free fatty acid which can be elongated to 18:0 and longer saturated fatty acids. While a small amount of this free fatty acid can reenter the pathway to 18:1$^{\Delta 9}$, much of it remains in the saturated fatty acid pathway and is directed as such into both storage and structural lipid.

Our results suggest a hitherto unrecognized control point that governs fatty acid composition. A mutation at this point could alter synthesis of saturated or unsaturated fatty acids. *Neurospora ufa* and *pfa* mutants (3–6), which are impaired in production of unsaturated fatty acids, have until now been presumed to have defects in desaturases, associated reductases, or in the synthesis of substrate lipids. These results, then, provide a new focal point for efforts in determining the regulation of fatty acid composition.

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TABLE 2
[^{2}H]-Fatty Acid Content of Each Fatty Acid Percentage[a]

Label position	16:0	18:0	18:1$^{\Delta 9}$	18:1$^{\Delta 9,12}$	18:1$^{\Delta 9,12,15}$	Long-chain	Minor unsaturates
[2,2-$^{2}H_2$]16:0	6.2	5.0	1.6	1.9	2.6	5.2	4.9
[16,16,16-$^{2}H_3$]16:0	6.9	4.6	No [^{2}H] detected	1.4	1.2	6.6	7.0

[a]Percentages of deuterated fatty acid for each fatty acid component of the total lipid, from 50 mL cultures grown with 30 µM palmitate deuterated in the 2,2- or 16,16,16-positions; results presented are the average of two determinations.

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